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Invention: INHIBITORS OF SERIES PROTEASE ACTIVITY OF MATRIPTASE OR MTSP1

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SPECIFICATION

DESCRIPTION

Inhibitors of Serine Protease Activity of Matriptase or MTSP1

Cross Reference to Related Applications

This application is a continuation-in-part of PCT Application No. PCT/US01/28137 filed September 7, 2001 which designates the United States of America, which is a continuation-in-part of United States Serial No. 09,675,986, filed September 8, 2000, the disclosures of which are incorporated herein by reference.

Field Of The Invention

The present invention is directed to methods of reducing tumor progression and/or metastasis. Matriptase and MTSP1 are serine proteases reported to be expressed in high levels in certain cancer cell lines.

According to one aspect, the present invention provides compounds which bind to the serine protease domain and are active as selective inhibitors of the serine protease activity of matriptase or MTSP1. These inhibitors can be used to inhibit the serine protease activity of matriptase or MTSP1 and thereby reduce harmful effects of its over-expression. Another aspect of the present invention is directed to the use of compounds that bind to the serine protease domain and selectively inhibit the serine protease activity of matriptase or MTSP1; such compounds may be used in the prevention and treatment of cancerous conditions and which decrease the growth of cancerous tumors and retard metastasis.

Background And Introduction To The Invention

Cancer is the second leading cause of death in the United States, after heart disease (Boring, C.C. et al., 1993, CA Cancer J. Clin. 43:7), and develops in one in three Americans. One of every four Americans dies of cancer. Cancer is characterized primarily by an increase in the number of abnormal, or neoplastic, cells derived from a given normal tissue which proliferate to form a tumor mass, the invasion of adjacent tissues by these neoplastic tumor cells, and the generation of malignant cells which spread via the blood or lymphatic system to regional lymph nodes and to distant sites. The latter progression to malignancy is referred to as metastasis.

Cancer can be viewed as a breakdown in the communication between tumor cells and their environment, including their normal neighboring cells. Signals, both growth-stimulatory and growth-inhibitory, are routinely exchanged between cells within a tissue. Normally, cells do not divide in the absence of stimulatory signals, and likewise, will cease dividing in the presence of inhibitory signals. In a cancerous, or neoplastic, state, a cell acquires the ability to "override" these signals and to proliferate under conditions in which normal cells would not grow.

Tumor cells must acquire a number of distinct aberrant traits to proliferate. Reflecting this requirement is the fact that the genomes of certain well-studied tumors carry several different independently altered genes, including activated oncogenes and inactivated tumor suppressor genes. Each of these genetic changes appears to be responsible for imparting some of the traits that, in aggregate, represent the full neoplastic phenotype (Land, H. et al. 1983, Science

222:771; Ruley, H.E., 1983, Nature 304:602; Hunter, T. 1991, cell 64:249).

In addition to unhindered cell proliferation, cells must acquire several traits for tumor progression to occur. For example, early on in tumor progression, cells must evade the host immune system. Further, as tumor mass increases, the tumor must acquire vasculature to supply nourishment and remove metabolic waste. Additionally, cells must acquire an ability to invade adjacent tissue, and, ultimately, cells often acquire the capacity to metastasize to distant sites.

A variety of biochemical factors have been associated with different phases of metastases. Cell surface receptors for collagen, glycoproteins such as laminin, or proteoglycans facilitate tumor cell attachment, an important step in invasion and metastases. Attachment then triggers the release of degradative enzymes which facilitate the penetration of tumor cells through tissue barriers. Once the tumor cells have entered the target tissue, specific growth factors are required for further proliferation.

One of the major characteristics of cancer cells is their ability to invade surrounding normal tissues and metastasize to distant body sites. It is the metastatic nature of malignant tumors that presents a great challenge to clinicians in terms of treatment, since the tumor is no longer localized to one area.

Tumor invasion (or progression) is a complex series of events, in which tumor cells detach from the primary tumor, break down the normal tissue surrounding it, and migrate into a blood or lymphatic vessel to be carried to a distant site. The breaking down of normal tissue barriers is accomplished by the elaboration of specific enzymes that degrade the proteins of the extracellular matrix that make up basement membranes and stromal components of tissues.

Elevated proteolytic activity has been implicated in neoplastic progression. The role(s) of proteolytic enzymes, including serine proteases, in neoplastic progression are under study. Proteases have been proposed to contribute to the degradation of excellular matrix and to tissue remodeling and, thus, may assist in cancer invasion and metastasis.

A number of extracellular proteases have been reported and expression of some of these proteases has been said to correlate with tumor progression. (Mignatti, P. and Rifkin, D.B., *Physiol. Rev.* 73:161-195 (1993).)

A class of extracellular matrix degrading enzymes has been identified called the matrix metalloproteinases (MMPs). Two of the matrix metalloproteinases have been implicated in tumor invasion. The type IV collagenase has been correlated with the metastatic potential of tumor cells. (Liotta, *et al.*, *Nature*, 284:67-68 (March, 1980)). It has been reported that the production of the matrix metalloproteinase stromelysin was associated with malignant tumors with metastatic potential. (McDonnell and Matrisian, Smnrs. in Cancer Biology 1:107-115 (1990); McDonnell and Matrisian, Cancer and Metastasis Reviews 9:309-319 (1990).)

The capacity of cancer cells to metastasize and invade tissue has been reported to be facilitated by degradation of the basement membrane. Several proteinase enzymes have been reported to facilitate the process of invasion of tumor cells. One family of enzymes, the MMPs, has been implicated as enhancing degradation of the basement membrane to allow tumorous cells to invade tissues. MMPs have been reported to differ in molecular weight and antigenic properties. Previously, two major metalloproteinases having molecular weights of about 70 kDa and 92 kDa have been studied. Both of these MMPs have been reported to enhance ability of tumor

cells to metastasize. Two natural inhibitors of these enzymes known as tissue inhibitors of metalloproteinase (TIMP) have been identified. The inactivated unclipped collagenases are generally secreted as a complex with TIMP. Enzymatic activity of the 72 kDa and 92 kDa proteins has been reported to depend on secreted ratios of collagenase/TIMP.

Matriptase is a trypsin-like serine protease which has been isolated and cloned from T-47D human breast cancer cells. Matriptase has been isolated from T-47D cell-conditioned medium. (Lin, *et al.*, *J. Biol. Chem.* 274(26):18231-18236 (1999).) Upon analysis of the cDNA, it was determined that the protease had 683 amino acids and contained three main structural regions: a serine protease domain near the carboxyl-terminal region, four tandem low-density lipoprotein receptor domains, and two tandem complement subcomponents, C1r and C1s. Matriptase was reported to be a mosaic protein with broad spectrum cleavage activity and two potential regulatory modules. It was named "matriptase" because of the ability of the protease to degrade the extra-cellular matrix and its trypsin-like activity. (Lin, *et al.*, *J. Bio. Chem.* 274:18231-18236 (1999).)

Matriptase is reported to be a protease having activity in degrading extracellular matrix that is localized on the cell surface. When isolated from breast cancer cells (or T-47D cell conditioned medium), matriptase has been reported to be primarily in an uncomplexed form. Matriptase has been isolated from human milk. When isolated from human milk, matriptase was reported to be in one of two complexed forms, 95 kDa (the predominant form) and 110 kDa; uncomplexed matriptase was not detected. (Liu, *et al.*, *J. Biol. Chem.* 274(26):18237-18242 (1999).) It has been proposed that

matriptase exists as an uncomplexed protease when in its active state. In breast milk, matriptase has been reported to exist in complex with a fragment of hepatocyte growth factor inhibitor-1 (HAI-1), a Kuntz-type serine protease inhibitor having activity against trypsin-like serine proteases.

Published PCT application WO 00/53232, "Matriptase, a Serine Protease and Its Applications", is said to describe matriptase.

Ecotin and Ecotin M84R/M85R have been reported to be macromolecular inhibitors of serine proteases of the chymotrypsin fold and have been reported to inhibit ductal branching, morphogenesis and differentiation of the explanted ductal prostate. PC-3 is a cell line derived from prostate cancer epithelial cells. Ecotin and M84R/M85R ecotin were found to decrease tumor size and metastasis in PC-3 implanted nude mice. Studies to identify additional serine proteases made by cancer cells were done using PC-3 cells. By using PCR techniques and degenerate oligonucleotide primers, five independent serine protease cDNAs were reported isolated from PC-3 mRNA. A serine protease termed "MT-SP1" was cloned, its cDNA characterized and reported to encode a mosaic, transmembrane protease. (Takeuchi, *et al.*, *PNAS* (US) 96:11054-11061 (1999).)

It was subsequently reported that the reported matriptase sequence was included in the translated sequence for the cDNA of MT-SP1. The matriptase cDNA was reported to be a partial MT-SP1 cDNA and to lack 516 of the coding nucleotides. (Takeuchi, *et al.*, *J. Biol. Chem* 275:26333-26342 (2000).) Since the reported matriptase cDNA sequence encoded a possible initiating methionine, it was proposed that alternative splicing could yield a protein lacking the N-terminal region of MT-SP1.

Both matriptase and MT-SP1 are reported to demonstrate trypsin-like protease activity. MT-SP1 has been reported to be a Type II transmembrane protein with an extracellular protease domain. Studies on matriptase have reported that a portion of enzyme molecules were localized on the surfaces of cells.

Additional studies have investigated the substrate specificity of MT-SP1. These experiments have reported that protease-activated receptor 2 (PAR2) and single-chain urokinase-type plasminogen activator (sc-uPA) are macromolecular substrates of MT-SP1. PAR2 is reported to function in inflammation, cytoprotection and/or cell adhesion, while sc-uPa is reported to function in tumor cell invasion and metastasis.

Published PCT application WO 01/57194, "Nucleic Acid Molecules Encoding Transmembrane Serine Proteases, the Encoded Proteins and Methods Based Thereon", is said to describe polypeptides which comprise protease domains of certain membrane-type serine proteases ("MTSP"), including MTSP1.

Published PCT application WO 99/42120 "TADG-15: An Extracellular Serine Protease Over expressed in Breast and Ovarian Carcenomas" and United States Patent No. 5,972,616 are said to describe DNA sequences encoding the TADG-15 protein and an isolated TADG-15 protein coded by such DNA sequences.

Published PCT application WO 01/29056 "TADG-15: An Extracellular Serine Protease Over expressed in Carcinomas" is said to describe DNA sequences encoding TADG-15 proteins.

Certain bis-benzamidine compounds which are said to be inhibitors of matriptase were reported by Enyedy, I.J. et al., *J. Med. Chem.* 44:1349-1355 (2001).

Published PCT application WO 01/97794 "Structure Based Discovery of Inhibitors of Matriptase for the Treatment of Cancer and Other Conditions", is said to describe methods of inhibiting carcinoma progression wherein matriptase plays a role and compounds useful in those methods.

Published PCT Application WO 02/08392 "Regulation of Human Matriptase-Like Serine Protease" is said to describe, *inter alia*, certain matriptase-like serine proteases, methods for their detection, and methods of screening for agents which modulate the activity of a human matriptase-like serine protease.

Summary of the Invention

In one aspect, the present invention relates to compounds which bind to the serine protease domain of matriptase or MTSP1 and which are selective inhibitors of the serine protease activity of matriptase or MTSP1 and to their use in inhibiting the serine protease activity matriptase or MTSP1.

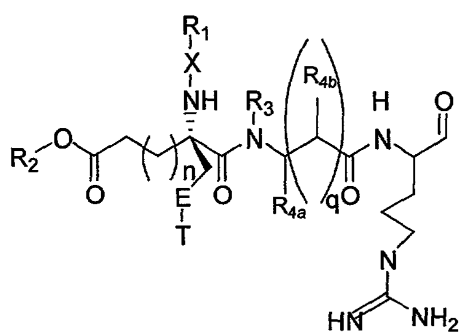
As noted above, the reported sequence for MTSP1 includes the reported matriptase sequence. We have cloned the serine protease domain and have determined that matriptase and MTSP1 have a common serine protease domain. Inhibition of serine protease activity of matriptase or MTSP1 is useful in the prevention and treatment of cancerous conditions, including having activity in retarding tumor progression and metastasis.

Matriptase and MTSP1 both have been reported to be trypsin-like serine proteases involved in the degradation of the extracellular matrix (ECM). Our cloning studies indicate that matriptase and MTSP1 share a common serine protease domain. Degradation of the extracellular matrix (basement membrane and interstitial stroma) is an important aspect of metastasis and is required for metastatic cancer

cells to migrate through anatomical barriers and to invade tissues. Thus, breakdown of the extracellular matrix is a primary factor in progression of cancerous conditions. In one aspect, the present invention relates to compounds and their use for the selective inhibition of serine protease activity of matriptase or MTSP1 in order to decrease the cancer-related breakdown of the extracellular matrix.

Accordingly to one aspect, the present invention provides compounds which are selective inhibitors of the serine protease activity of matriptase or MTSP1. Certain compounds having activity in inhibiting the serine protease activity of matriptase or MTSP1 are depicted in Figures 1A to 1C.

According to an alternate aspect of the present invention, provided are novel compounds of formula (I):



(I)

wherein:

(a) X is selected from the group consisting of -C(=O)-, -C(=O)-O-, -C(=O)NH-, $-S(O)_2-$, $-S(O)_2NH-$ and a direct link;

(b) R_1 is selected from the group consisting of

(1) alkyl of 1 to about 12 carbon atoms which is unsubstituted or substituted with 1 or 2 substituents selected from the group consisting of Y_1 and Y_2;

(2) alkyl of 1 to about 3 carbon atoms substituted with cycloalkyl of about 3 to about 8 carbon atoms which is unsubstituted or substituted with 1 to 3 substituents selected from the group consisting of Y_1, Y_2 and Y_3;

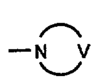
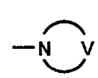
(3) cycloalkyl of 3 to about 8 carbon atoms which is unsubstituted or substituted with 1 to 3 substituents selected from the group consisting of Y_1, Y_2 and Y_3; and

(4) aryl of about 6 to 14 carbon atoms which is unsubstituted or substituted with 1 to 3 substituents selected from the group consisting of Y_1, Y_2 and Y_3;

(5) aralkyl of about 7 to about 15 carbon atoms which is unsubstituted or substituted on the alkyl chain with hydroxy or halogen and which is unsubstituted or substituted on the aryl ring with 1 to 3 substituents selected from the group consisting of Y_1, Y_2 and Y_3;

(6) hydrogen when X is $-C(=O)NH-$, $-S(O)_2-$, $-S(O)_2NH-$, or a direct link;

(7) heterocycloalkyl of 4 to about 10 ring atoms with the ring atoms selected from carbon and heteroatoms, wherein the heteroatoms are selected from the group consisting of oxygen, nitrogen, and S(O)i, wherein i is 0, 1 or 2, which is unsubstituted or mono-, di-, or tri-substituted on the ring with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

(8) heterocyclo of 4 to about 10 ring atoms with the ring atoms selected from carbon and heteroatoms, wherein the heteroatoms are selected from the group consisting of oxygen, nitrogen, and $S(O)_i$, wherein i is 0, 1, or 2, including,  wherein  is a 5 to 7 member heterocycle having 3 to 6 ring carbon atoms, where V is $-CH_2-$, $-O-$, $-S(=O)-$, $-S(O)_2-$ or $-S-$, which is unsubstituted or mono-, di-, or tri-substituted on the ring carbons with 1 to

3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

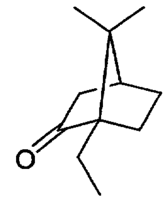
(9) alkenyl of 2 to about 6 carbon atoms which is unsubstituted or substituted with cycloalkyl of about 3 to about 8 carbon atoms, which is unsubstituted or mono-, di-, or tri-substituted on the ring with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

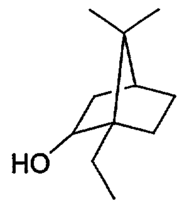
(10) heteroaryl of about 5 to about 14 ring atoms with the ring atoms selected from carbon and heteroatoms, wherein the heteroatoms are selected from oxygen, nitrogen, and sulfur, and which is unsubstituted or mono-, di- or tri-substituted with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

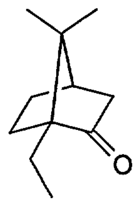
(11) heteroaralkyl of about 5 to about 14 ring atoms with the ring atoms selected from carbon and heteroatoms, wherein the heteroatoms are selected from oxygen, nitrogen, and sulfur, which is unsubstituted or substituted on the alkyl chain with hydroxy or halogen and which is unsubstituted on the ring or mono-, di- or tri-substituted on the ring with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

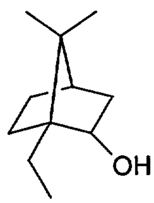
(12) aralkenyl of about 8 to about 16 carbon atoms which is unsubstituted or mono-, di-, or tri-substituted on the aryl ring with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

(13) heteroaralkenyl of about 5 to about 14 ring atoms with the ring atoms selected from carbon and heteroatoms, wherein the heteroatoms are selected from oxygen, nitrogen, and sulfur, and which is unsubstituted or mono-, di- or tri-substituted on the ring carbons with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

(14)  ;

(15)  ;

(16)  ;

(17)  ;

(18) fused carbocyclic alkyl of about 9 to about 15 carbon atoms;

(19) difluoromethyl or perfluoroalkyl of 1 to about 12 carbon atoms;

(20) perfluoroaryl of about 6 to about 14 carbon atoms; and

(21) perfluoroaralkyl of about 7 to about 15 carbon atoms., and

wherein each Y_1, Y_2, and Y_3 is independently selected and is

(i) selected from the group consisting of halogen, cyano, nitro, tetrazolyl, guanidino, amidino, methylguanidino, $-CF_3$, $-CF_2CF_3$, $-CH(CF_3)_2$, $-C(OH)(CF_3)_2$, $-OCF_3$, $-OCF_2H$, $-OCF_2CF_3$, $-OC(O)NH_2$, $-OC(O)NHZ_1$, $-OC(O)NZ_1Z_2$, $-NHC(O)Z_1$, $-NHC(O)NH_2$, $-NHC(O)NZ_1$, $-NHC(O)NZ_1Z_2$, $-C(O)OH$, $-C(O)OZ_1$, $-C(O)NH_2$, $-C(O)NHZ_1$, $-C(O)NZ_1Z_2$, $-P(O)_3H_2$, $-P(O)_3(Z_1)_2$, $-S(O)_3H$, $-S(O)_mZ_1$, $-Z_1$, $-OZ_1$, $-OH$, $-NH_2$, $-NHZ_1$,

$-NZ_1Z_2$, $-C(=NH)NH_2$, $-C(=NOH)NH_2$, -N-morpholino, and $-S(O)_m(CF_2)_qCF_3$, wherein m is 0, 1 or 2, q is an integer from 0 to 5, and Z_1 and Z_2 are independently selected from the group consisting of alkyl of 1 to about 12 carbon atoms, aryl of about 6 to about 14 carbon atoms, heteroaryl of about 5 to about 14 ring atoms, aralkyl of about 7 to about 15 carbon atoms, and heteroaralkyl of about 5 to about 14 ring atoms, or

(ii) Y_1 and Y_2 are selected together to be $-O[C(Z_3)(Z_4)]_rO-$ or $-O[C(Z_3)(Z_4)]_{r+1}-$, wherein r is an integer from 1 to 4 and Z_3 and Z_4 are independently selected from the group consisting of hydrogen, alkyl of 1 to about 12 carbon atoms, aryl of about 6 to about 14 carbon atoms, heteroaryl of about 5 to about 14 ring atoms, aralkyl of about 7 to about 15 carbon atoms, and heteroaralkyl of about 5 to about 14 ring atoms;

(c) R_2 is hydrogen or alkyl of 1 to about 12 carbon atoms;

(d) n is 0, 1, 2 or 3;

(e) R_3 is hydrogen or methyl or R_3, R_{4a} and q are selected together as set forth in (g);

(f) R_{4a} and R_{4b} are independently hydrogen, lower alkyl of 1 to about 3 carbon atoms, and q is 0, 1 or 2, or R_3, R_{4a} and q are selected together as set forth in (g);

(g) q is 0 and R_3 and R_{4a} are selected together to be in the S-configuration to give a group at P2 selected from the group consisting of prolyl, pipecolyl, azetidine-2-carbonyl; 4-hydroxyprolyl, 3-hydroxyprolyl, 4-aminoprolyl, 4-(CH_2NH_2)-prolyl, 3,4-methanoprolyl and 3,4-dehydroprolyl; and

(h) E is a 5- or 6- membered aromatic ring having 0 to 2 ring heteroatoms and the remainder of the ring atoms carbon atoms, wherein the heteratoms are selected from the

group consisting of oxygen, nitrogen and sulfur, and which is substituted with R_5 and R_6 wherein R_5 and R_6 are independently selected from the group consisting of hydogen, hydroxy, halogen, alkyl of 1 to about 6 carbon atoms, alkyl of 1 to about 4 carbon atoms substituted with alkoxy of 1 to about 4 carbon atoms, alkoxy of 1 to about 6 carbon atoms and trifluoromethyl;

(i) T is hydrogen, hydroxy, $-CH_2OH$, alkyl of 1 to about 3 carbon atoms, cyano, $-C(=NR_7)NHR_8$, $-NH-C(=NR_7)NHR_8$, $-NHR_9$ or $-C(=O)NHR_9$ wherein R_7 and R_8 are independently hydrogen, hydroxy, alkoxy of 1 to about 3 carbon atoms, trihydrocarbosilyl of 3 to about 16 carbon atoms, alkyl of 1 to about 3 carbon atoms or $-C(=O)R_9$ wherein R_9 is hydrogen, alkyl of 1 to about 6 carbon atoms, alkoxy of 1 to about 6 carbon atoms or $-(CF_2)_jCF_3$ wherein j is 0, 1, 2 or 3, and with the proviso that R_7 and R_8 are not both hydroxy or alkoxy; and pharmaceutically acceptable salts thereof. These compounds have activity as selective inhibitors of the serine protease activity of matriptase or MTSP1.

Also, according to an aspect of the present invention, provided are pharmaceutical compositions which comprise an amount effective to inhibit or decrease serine protease activity of matriptase or MTSP1 of a compound of formula I.

According to an alternate aspect of the present invention, provided are methods of creating a pathologic condition in a mammal which is ameliorated by decreasing or inhibiting serine protease activity of metriptase or MTSP1 which comprises administering to said mammal an amount of a compound of formula I effective to decrease or inhibit serine protease activity of matriptase or MTSP1.

Also provided are methods of treating a pathologic condition in mammal which is ameliorated by decreasing or inhibiting serine protease activity of matriptase or MTSP1

which comprises administering to said mammal a pharmaceutical composition comprising a compound of formula effective to decrease or inhibit serine protease activity of matriptase or MTSP1.

The present invention is also directed to administration of a selective inhibitor of serine protease activity of matriptase or MTSP1 to an area of a mammal suspected of having metastic cancer cells in order to retard tumor progression. Also, provided are pharmaceutical compositions which comprise a selective inhibitor of serine protease activity matriptase or MTSP1 and a pharmaceutically acceptable carrier.

Definitions

"Matriptase" is a trypsin-like serine protease active in the development of cancerous conditions, such as tumors and metastasis of cancer. Matriptase is a matrix degrading protease that is reported to be localized on the cell surface. Matriptase is reported to have 683 amino acids and contains three main structural regions, a serine protease domain near the carboxyl-terminal region, four tandem low-density lipoprotein receptor domains, and two tandem complement subcomponents C1r and C1s.

Membrane-type serine protease 1 ("MT-SP1" or "MTSP1") refers to a serine protease originally cloned from the PC-3 cell line. MT-SP1 is reported to be a Type II transmembrane protein with an extracellular protease domain. The amino acid sequence reported for matriptase has been reported to be included within the translated sequence for the cDNA of MT-SP1. The cDNA of MT-SP1 is reported to have, in addition to regions coding for the three main structural regions described above for matriptase, an additional 516 nucleotides that make up a signal/anchor domain.

A "cancerous condition" is one in which the patient has a progressive human cancer, such as, leukemia, lymphomas, human melanomas, breast, gastrointestinal, such as esophageal, stomach, colon, bowel, colorectal and rectal cancers, prostate, bladder, testicular, ovarian, uterine, cervical, brain, lung, bronchial, larynx, pharynx, pancreatic, thyroid, bone, and various types of skin cancers.

"Metastasis" is the spread of cancer from an original location to a new location in the body.

In accordance with the present invention and as used herein, the following terms are defined to have following meanings, unless explicitly stated otherwise:

The term "alkenyl" refers to unsaturated aliphatic groups having at least one double bond.

The term "alkyl" refers to saturated aliphatic groups including straight-chain, branched-chain and cyclic (including polycyclic) groups.

The terms "alkoxy" and "alkoxyl" refer to a group having the formula, R-O-, wherein R is an alkyl group.

The term "alkoxycarbonyl" refers to -C(O)OR wherein R is alkyl.

The term "aralkenyl" refers to an alkenyl group substituted with an aryl group. Preferably the alkenyl group has from 2 to about 6 carbon atoms.

The term "aralkyl" refers to an alkyl group substituted with an aryl group. Suitable aralkyl groups include benzyl, phenethyl, and the like, all of which may be optionally substituted. Preferably the alkyl group has from 1 to about 6 carbon atoms.

The term "aryl" refers to an aromatic group which has at least one ring having a conjugated pi electron system and

includes a carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted.

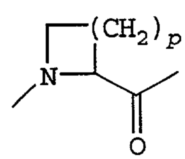
The term "aryloxy" refers to a group having the formula, R-O-, wherein R is an aryl group.

The term "aralkoxy" refers to a group having the formula, R-O-, wherein R is an aralkyl group.

The term "amino acid" refers to both natural, unnatural amino acids in their D and L stereo isomers if their structures allow such stereoisomeric forms, and their analogs. Natural amino acids include alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val). Unnatural amino acids include, but are not limited to azetidinecarboxylic acid, 2-aminoadipic acid, 3-aminoadipic acid, beta-alanine, aminopropionic acid, 2-aminobutyric acid, 4-aminobutyric acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisobutyric acid, 2-aminopimelic acid, 2,4 diaminoisobutyric acid, demosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, hydroxylysine, allo-hydroxylysine, 3-hydroxyproline, 4-hydroxyproline, isodesmosine, allo-isoleucine, N-methylglycine, N-methylisoleucine, N-methylvaline, norvaline, norleucine, ornithine and pipecolic acid. Amino acid analogs include the natural and unnatural amino acids which are chemically blocked, reversibly or irreversibly, or modified on their N-terminal amino group or their side-chain groups, as for example, methionine sulfoxide, methionine sulfone,

S-(carboxymethyl)-cysteine, S-(carboxymethyl)-cysteine sulfoxide and S-(carboxymethyl)-cysteine sulfone.

The term "amino acid analog" refers to an amino acid wherein either the C-terminal carboxy group, the N-terminal amino group or side-chain functional group has been chemically modified to another functional group. For example, aspartic acid-(beta-methyl ester) is an amino acid analog of aspartic acid; N-ethylglycine is an amino acid analog of glycine; or alanine carboxamide is an amino acid analog of alanine.

The term "amino acid residue" refers to radicals having the structure: (1) -C(O)-R-NH-, wherein R typically is -CH(R')-, wherein R' is H or a carbon containing substituent; or (2) 

, wherein p is 1, 2 or 3 representing the azetidinecarboxylic acid, proline or pipecolic acid residues, respectively.

"Biaryl" refers to phenyl substituted by carbocyclic or heterocyclic aryl as defined herein, ortho, meta or para to the point of attachment of the phenyl ring.

"Brine" refers to an aqueous saturated solution of sodium chloride.

"Carbocyclic aryl" refers to aromatic groups wherein the ring atoms on the aromatic ring are carbon atoms. Carbocyclic aryl groups include monocyclic carbocyclic aryl groups and naphthyl groups, all of which may be optionally substituted. Suitable carbocyclic aryl groups include phenyl and naphthyl. Suitable substituted carbocyclic aryl groups include indene and phenyl substituted by one to two substituents such being advantageously lower alkyl, hydroxy, lower alkoxy, lower alkoxycarbonyl, halogen, trifluoromethyl, difluoromethyl, nitro, and cyano.

Substituted naphthyl refers to naphthyl, more preferably 1- or 2-naphthyl, substituted by Y_1, Y_2 and/or Y_3 as defined in connection with formula (I) hereinabove.

"Cycloalkenyl" refers to a cyclic alkenyl group. Suitable cycloalkenyl groups include, for example, cyclopentenyl and cyclohexenyl.

"Cycloalkyl" refers to a cyclic alkyl group having at least one ring and includes polycyclic groups, including fused ring cyclic alkyl groups. Suitable cycloalkyl groups include, for example, cyclohexyl, cyclopropyl, cyclopentyl, and cycloheptyl.

"Cyclohexylmethyl" refers to a cyclohexyl group attached to $-CH_2-$.

"Fused carbocyclic" refers to a multicyclic fused carbocyclic ring having both aromatic and non-aromatic rings. Suitable fused carbocyclic rings include fluorenyl, tetralin and the like.

"Fused carbocyclic alkyl" refers to an alkyl group substituted with a fused carbocyclic ring moiety, preferably a multicyclic fused carbocyclic ring including both aromatic and non-aromatic rings. Suitable fused carbocyclic alkyl groups include fluorenylmethyl, and the like.

The term "halogen" refers to fluorine, chlorine, bromine and iodine.

"Heteroaralkenyl" refers to an alkenyl group substituted with a heteroaryl, and includes those heterocyclic systems described in "Handbook of Chemistry and Physics", 49th edition, 1968, R.C. Weast, editor; The Chemical Rubber Co., Cleveland, OH. See particularly Section C, Rules for Naming Organic Compounds, B. Fundamental Heterocyclic Systems. Preferably the alkenyl group has from 2 to about 6 carbon atoms.

"Heteroaralkyl" refers to an alkyl group substituted with a heteroaryl, such as picolyl, and includes those heterocyclic systems described in "Handbook of Chemistry and Physics", 49th edition, 1968, R.C. Weast, editor; The Chemical Rubber Co., Cleveland, OH. See particularly Section C, Rules for Naming Organic Compounds, B. Fundamental Heterocyclic Systems. Preferably the alkyl group has from 1 to about 6 carbon atoms.

"Heteroaryl" refers to aromatic groups having from 1 to 14 carbon atoms and the remainder of the ring atoms are heteroatoms, and includes those heterocyclic systems described in "Handbook of Chemistry and Physics", 49th edition, 1968, R.C. Weast, editor; The Chemical Rubber Co., Cleveland, OH. See particularly Section C, Rules for Naming Organic Compounds, B. Fundamental Heterocyclic Systems. Suitable heteroatoms include oxygen, nitrogen, and $S(O)_i$, wherein i is 0, 1 or 2, and suitable heterocyclic aryls include furanyl, thienyl, pyridyl, pyrrolyl, pyrimidyl, pyrazinyl, imidazolyl, and the like.

"Heterocyclo" refers to a reduced heterocyclic ring system comprised of carbon, nitrogen, oxygen and/or sulfur atoms, and includes those heterocyclic systems described in "Handbook of Chemistry and Physics", 49th edition, 1968, R.C. Weast, editor; The Chemical Rubber Co., Cleveland, OH. See particularly Section C, Rules for Naming Organic Compounds, B. Fundamental Heterocyclic Systems.

"Heterocycloalkyl" refers to an alkyl group substituted with a heterocyclo group, and includes those heterocyclic systems described in "Handbook of Chemistry and Physics", 49th edition, 1968, R.C. Weast, editor; The Chemical Rubber Co., Cleveland, OH. See particularly Section C, Rules for Naming Organic Compounds, B. Fundamental Heterocyclic

Systems. Preferably the alkyl group has from about 1 to about 6 carbon atoms.

The term "lower" referred to herein in connection with organic radicals or groups defines such radicals or groups with one and up to and including 5 carbon atoms, preferably up to and including 4 carbon atoms, and advantageously one or two carbon atoms. Such radicals or groups may be straight chain or branched chain.

"Perfluoroalkyl" refers to an alkyl group which has every hydrogen replaced with fluorine.

"Perfluoroaryl" refers to an aryl group which has every hydrogen replaced with fluorine.

"Perfluoroarylalkyl" refers to an aralkyl group in which every hydrogen on the aryl moiety is replaced with fluorine.

"Pharmaceutically acceptable salt" includes salts of the compounds of the present invention derived from the combination of such compounds and an organic or inorganic acid. In practice the use of the salt form amounts to use of the base form. The compounds of the present invention are useful in both free base and salt form, with both forms being considered as being within the scope of the present invention.

In addition, the following abbreviations stand for the following:

"AcN", CH_3CN or "MeCN" refer to acetonitrile.

"AIBN" refers to 2, 2'-azobisisobutyronitrile.

"Bn" refers to benzyl.

"Boc" refers to t-butoxycarbonyl.

"Boc_2O" refers to Boc anhydride (di-tert-butyl carbonate).

"BOC-ON" refers to 2-(tert-butoxycarbonyloxyamino)-2-phenylacetonitrile.

"$BzlSO_2$" refers to benzylsulfonyl.

"Cbz" or "CBz" refers to benzyloxycarbonyl.

"$CNNH_2$" or "H_2NCN" refers to cyanamide.

"$CsCO_3$" refers to cesium carbonate.

"DCA" refers to dichloroacetic acid.

"DCC" refers to N,N'-dicyclohexylcarbodiimide.

"DCM"or "CH_2Cl_2" refers to dichloromethane.

"DIEA" refers to diisopropylethylamine.

"DMF" refers to N,N-dimethylformamide.

"DMSO" refers to dimethyl sulfoxide.

"DMAP" refers to 4-N,N-dimethylaminopyridine.

"EDC" refers to 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride salt.

"Et_3N" or "TEA" refers to triethylamine.

"EtOAc" refers to ethyl acetate.

"EtOH" refers to ethanol.

"HATU" refers to O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluromium hexafluorophosphate.

"HBTU" refers to 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

"HCl" refers to hydrochloric acid.

"HOAc" refers to acetic acid.

"HOAt" or "HOAT" refers to 1-hydroxy-7-azabenzotriazole.

"HOBt" refers to 1-hydroxybenzotriazole monohydrate.

"i-BuOCOCl" refers to isobutylchloroformate.

"HPLC" refers to high pressure liquid chromatography.

"KHMDS" refers to potassium hexamethyldisilazide, also termed potassium bis(trimethylsilyl)amide.

"$LiAlH_4$" refers to lithium aluminum hydride.

"$LiAlH_2(OEt)_2$" refers to lithium aluminum hydride diethoxide.

"Me" refers to methyl.

"MeOH" refers to methanol.

"NMM" refers to N-methylmorpholine.

"NBS" refers to N-bromosuccinimide.

"$PhB(OH)_2$" refers to phenylboronic acid.

"Ph_3P" or "PPh_3" refers to triphenylphospine.

"PyBOP" refers to benzotriazole-ly-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate.

"RP-HPLC" refers to reverse phase high pressure liquid chromatography.

"Swern Ox" refers to a reaction step called a "Swern oxidation", see Example 6.

"TFA" refers to trifluoroacetic acid.

"THF" refers to tetrahydrofuran.

"TLC" refers to thin layer chromatography.

Brief Description of the Figures

Figures 1A to 1C depict certain preferred selective inhibitors of matriptase.

Figures 2A and 2B depict a reaction scheme for the synthesis of compound 6 of Figure 1A.

Figures 3A to 3C depict the nucleic acid sequence (top line) (SEQ. ID. NO. 1) and corresponding amino acid sequence (SEQ. ID. NO. 2) for a cDNA fragment encoding the entire serine protease domain of recombinant matriptase or MTSP1 (rMAP) cloned from the human adenocarcinoma cell line PC-3 as described in Example 1. The second line of nucleotides is the complementary sequence (SEQ. ID. NO. 11) to SEQ. ID. NO. 1.

Figures 4A and 4B depict a reaction scheme for the synthesis of a compound of the present invention. This reaction scheme is further described in Examples 3 to 13.

Figures 5A and 5B depict certain inhibitors of the serine protease activity of Matriptase or MTSP1.

Detailed Description of the Invention

1. Preferred Compounds

Suitable compounds for use according to the methods of the present invention are selected for their potency and selectivity in inhibiting serine protease activity of matriptase or MTSP1 while not significantly inhibiting activity of other serine proteases important for cellular homeostasis.

Preferred compounds for use according to the present invention include those which will selectively inhibit serine protease activity of matriptase or MTSP1 without substantially inhibiting circulating serine proteases, particularly serine proteases of the coagulation cascade, especially thrombin and factor Xa.

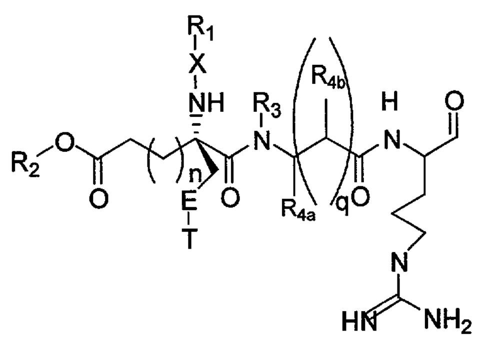
In one aspect, preferred are compounds which selectively inhibit the serine protease activity of matriptase or MTSP1 but which do not appreciably inhibit the activity of factor Xa or thrombin. More preferably, such compounds do not appreciably inhibit both factor Xa and thrombin. These compounds are characterized by having a IC_{50} for either factor Xa or thrombin which is at least two times, and more preferably five times greater, than its IC_{50} for inhibiting serine protease activity of matriptase or MTSP1. Preferably, the compound will have an IC_{50} for each of factor Xa and thrombin at least two times and more preferably five times or greater than its IC_{50} for matriptase or MTSP1. More preferred are those compounds having an IC_{50} for each of factor Xa and thrombin that is at an order of magnitude (10 times) higher than the IC_{50} for serine protease activity of matriptase or MTSP1. More preferred are those compounds which also do not appreciably inhibit plasmin, and which are characterized by having an IC_{50} for plasmin which is at least two times, more preferably five times, their IC_{50}

for serine protease activity of matriptase or MTSP1. These compounds are believed to be useful either as *in vitro* diagnostic agents for selectively inhibiting serine protease activity of matriptase or MTSP1 while only weakly inhibiting, if at all, factor Xa, thrombin and/or plasmin or as pharmacological agents for the treatment of pathologic disorders characterized by over-expression and/or over-activity of serine protease activity matriptase or MTSP1.

According to an alternate aspect of the present invention, preferred compounds include compounds which inhibit serine protease activity of matriptase or MTSP1 by a non-covalent mode of action.

According to one aspect of the present invention, compounds which are inhibitors of serine protease activity matriptase or MTSP1 include those depicted in Figures 1A to 1C.

According to an alternate aspect provided are compounds of formula (I):



(I)

wherein:

(a) X is selected from the group consisting of -C(=O)-, -C(=O)-O-, -C(=O)NH-, $-S(O)_2-$, $-S(O)_2NH-$ and a direct link;

(b) R_1 is selected from the group consisting of

(1) alkyl of 1 to about 12 carbon atoms which is unsubstituted or substituted with 1 or 2 substituents selected from the group consisting of Y_1 and Y_2;

(2) alkyl of 1 to about 3 carbon atoms substituted with cycloalkyl of about 3 to about 8 carbon atoms which is unsubstituted or substituted with 1 to 3 substituents selected from the group consisting of Y_1, Y_2 and Y_3;

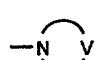
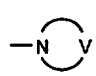
(3) cycloalkyl of 3 to about 8 carbon atoms which is unsubstituted or substituted with 1 to 3 substituents selected from the group consisting of Y_1, Y_2 and Y_3; and

(4) aryl of about 6 to 14 carbon atoms which is unsubstituted or substituted with 1 to 3 substituents selected from the group consisting of Y_1, Y_2 and Y_3;

(5) aralkyl of about 7 to about 15 carbon atoms which is unsubstituted or substituted on the alkyl chain with hydroxy or halogen and which is unsubstituted or substituted on the aryl ring with 1 to 3 substituents selected from the group consisting of Y_1, Y_2 and Y_3;

(6) hydrogen when X is $-C(=O)NH-$, $-S(O)_2-$, $-S(O)_2NH-$, or a direct link;

(7) heterocycloalkyl of 4 to about 10 ring atoms with the ring atoms selected from carbon and heteroatoms, wherein the heteroatoms are selected from the group consisting of oxygen, nitrogen, and $S(O)_i$, wherein i is 0, 1 or 2, which is unsubstituted or mono-, di-, or tri-substituted on the ring with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

(8) heterocyclo of 4 to about 10 ring atoms with the ring atoms selected from carbon and heteroatoms, wherein the heteroatoms are selected from the group consisting of oxygen, nitrogen, and $S(O)_i$, wherein i is 0, 1, or 2, including,  wherein  is a 5 to 7 member

heterocycle having 3 to 6 ring carbon atoms, where V is $-CH_2-$, -O-, -S(=O)-, $-S(O)_2-$ or -S-, which is unsubstituted or mono-, di-, or tri-substituted on the ring carbons with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

(9) alkenyl of 2 to about 6 carbon atoms which is unsubstituted or substituted with cycloalkyl of about 3 to about 8 carbon atoms, which is unsubstituted or mono-, di-, or tri-substituted on the ring with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

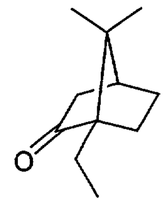
(10) heteroaryl of about 5 to about 14 ring atoms with the ring atoms selected from carbon and heteroatoms, wherein the heteroatoms are selected from oxygen, nitrogen, and sulfur, and which is unsubstituted or mono-, di- or tri-substituted with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

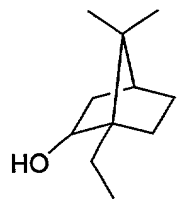
(11) heteroaralkyl of about 5 to about 14 ring atoms with the ring atoms selected from carbon and heteroatoms, wherein the heteroatoms are selected from oxygen, nitrogen, and sulfur, which is unsubstituted or substituted on the alkyl chain with hydroxy or halogen and which is unsubstituted on the ring or mono-, di- or tri-substituted on the ring with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

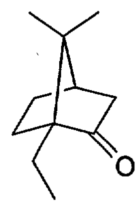
(12) aralkenyl of about 8 to about 16 carbon atoms which is unsubstituted or mono-, di-, or tri-substituted on the aryl ring with 1 to 3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

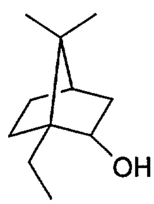
(13) heteroaralkenyl of about 5 to about 14 ring atoms with the ring atoms selected from carbon and heteroatoms, wherein the heteroatoms are selected from oxygen, nitrogen, and sulfur, and which is unsubstituted or mono-, di- or tri-substituted on the ring carbons with 1 to

3 substituents selected from the group consisting of Y_1, Y_2, and Y_3;

(14)  ;

(15)  ;

(16)  ;

(17)  ;

(18) fused carbocyclic alkyl of about 9 to about 15 carbon atoms;

(19) difluoromethyl or perfluoroalkyl of 1 to about 12 carbon atoms;

(20) perfluoroaryl of about 6 to about 14 carbon atoms; and

(21) perfluoroaralkyl of about 7 to about 15 carbon atoms., and

wherein each Y_1, Y_2, and Y_3 is independently selected and is

(i) selected from the group consisting of halogen, cyano, nitro, tetrazolyl, guanidino, amidino, methylguanidino, $-CF_3$, $-CF_2CF_3$, $-CH(CF_3)_2$, $-C(OH)(CF_3)_2$, $-OCF_3$, $-OCF_2H$, $-OCF_2CF_3$, $-OC(O)NH_2$, $-OC(O)NHZ_1$, $-OC(O)NZ_1Z_2$, $-NHC(O)Z_1$, $-NHC(O)NH_2$, $-NHC(O)NZ_1$, $-NHC(O)NZ_1Z_2$, $-C(O)OH$,

$-C(O)OZ_1$, $-C(O)NH_2$, $-C(O)NHZ_1$, $-C(O)NZ_1Z_2$, $-P(O)_3H_2$, $-P(O)_3(Z_1)_2$, $-S(O)_3H$, $-S(O)_mZ_1$, $-Z_1$, $-OZ_1$, -OH, $-NH_2$, $-NHZ_1$, $-NZ_1Z_2$, $-C(=NH)NH_2$, $-C(=NOH)NH_2$, -N-morpholino, and $-S(O)_m(CF_2)_qCF_3$, wherein m is 0, 1 or 2, q is an integer from 0 to 5, and Z_1 and Z_2 are independently selected from the group consisting of alkyl of 1 to about 12 carbon atoms, aryl of about 6 to about 14 carbon atoms, heteroaryl of about 5 to about 14 ring atoms, aralkyl of about 7 to about 15 carbon atoms, and heteroaralkyl of about 5 to about 14 ring atoms, or

(ii) Y_1 and Y_2 are selected together to be $-O[C(Z_3)(Z_4)]_rO-$ or $-O[C(Z_3)(Z_4)]_{r+1}-$, wherein r is an integer from 1 to 4 and Z_3 and Z_4 are independently selected from the group consisting of hydrogen, alkyl of 1 to about 12 carbon atoms, aryl of about 6 to about 14 carbon atoms, heteroaryl of about 5 to about 14 ring atoms, aralkyl of about 7 to about 15 carbon atoms, and heteroaralkyl of about 5 to about 14 ring atoms;

(c) R_2 is hydrogen or alkyl of 1 to about 12 carbon atoms;

(d) n is 0, 1, 2 or 3;

(e) R_3 is hydrogen or methyl or R_3, R_{4a} and q are selected together as set forth in (g);

(f) R_{4a} and R_{4b} are independently hydrogen, lower alkyl of 1 to about 3 carbon atoms, and q is 0, 1 or 2, or R_3, R_{4a} and q are selected together as set forth in (g);

(g) q is 0 and R_3 and R_{4a} are selected together to be in the S-configuration to give a group at P2 selected from the group consisting of prolyl, pipecolyl, azetidine-2-carbonyl; 4-hydroxyprolyl, 3-hydroxyprolyl, 4-aminoprolyl, 4-(CH_2NH_2)-prolyl, 3,4-methanoprolyl and 3,4-dehydroprolyl; and

(h) E is a 5- or 6- membered aromatic ring having 0 to 2 ring heteroatoms and the remainder of the ring atoms carbon atoms, wherein the heteratoms are selected from the group consisting of oxygen, nitrogen and sulfur, and which is substituted with R_5 and R_6 wherein R_5 and R_6 are independently selected from the group consisting of hydogen, hydroxy, halogen, alkyl of 1 to about 6 carbon atoms, alkyl of 1 to about 4 carbon atoms substituted with alkoxy of 1 to about 4 carbon atoms, alkoxy of 1 to about 6 carbon atoms and trifluoromethyl;

(i) T is hydrogen, hydroxy, $-CH_2OH$, alkyl of 1 to about 3 carbon atoms, cyano, $-C(=NR_7)NHR_8$ $-NH-C(=NR_7)NHR_8$, $-NHR_9$ or $-C(=O)NHR_9$ wherein R_7 and R_8 are independently hydrogen, hydroxy, alkoxy of 1 to about 3 carbon atoms, trihydrocarbosilyl of 3 to about 16 carbon atoms, alkyl of 1 to about 3 carbon atoms or $-C(=O)R_9$ wherein R_9 is hydrogen, alkyl of 1 to about 6 carbon atoms, alkoxy of 1 to about 6 carbon atoms or $-(CF_2)_jCF_3$ wherein j is 0, 1, 2 or 3, and with the proviso that R_7 and R_8 are not both hydroxy or alkoxy; and pharmaceutically acceptable salts thereof. These compounds have activity in inhibiting the serine protease activity of matriptase or MTSP1.

According to this aspect of the present invention, examples of substituents are set forth below.

Exemplary X groups include a direct link, $-C(=O)-O-$, $-C(O)NH-$, and $-S(O)_2-$.

Exemplary R_1 groups include R_1 groups of paragraph (b)(1) to (b)(6) as set forth hereinabove.

Exemplary R_2 groups include hydrogen and lower alkyl of 1 to about 3 carbon atoms.

Exemplary values for q include 0 and 1, more preferably q is 0.

Exemplary values for n include 0 and 1, more preferably n is 1.

Exemplary R_3 groups include hydrogen or R_3, R_{4a} and q are selected together as set forth in paragraph (g) hereinabove.

Exemplary R_{4a} and R_{4b} groups include hydrogen and methyl or q, R_3 and R_{4a} are selected together as set forth in paragraph (g) hereinabove.

Exemplary E groups include aromatic rings such as phenyl, thiophene, pyridine, pyrimidine, pyrazine, pyridazine, imidazole, thiazole, oxazole and pyrazole.

Exemplary T groups include hydrogen, methyl, cyano, amidino, guanidino and amino. According to one embodiment of this aspect of the present invention, preferred T groups include amidino.

According to one embodiment of this aspect of this invention, provided are compounds of formula I wherein q is zero. Preferably n is 0 or 1. Preferably R_3 is hydrogen or methyl. Preferably R_2 is hydrogen or lower alkyl of 1 to about 3 carbon atoms; preferred lower alkyl groups include methyl. Preferably X is a direct link, $-S(O)_2-$ $-C(=O)-O-$ or $-C(=O)NH-$. When X is a direct link, R_1 is preferably hydrogen. When X is $-S(O)_2-$, R_1 is preferably aryl. When X is $-C(=O)-O-$, R_1 is preferably alkyl. When X is $C(=O)NH-$, R_1 is preferably alkyl. Preferably E is phenyl. Especially preferred T groups include $C(=NR_7)NHR_8$, more preferably R_7 and R_8 are hydrogen.

According to another embodiment of this aspect of the present invention, provided are compounds of formula I wherein q, R_3 and R_{4a} are selected together as set forth in paragraph (g) hereinabove.

According to another embodiment of this aspect of the invention, provided are compounds of formula I wherein q is 0 and E is phenyl. Preferably X is a direct link and R_1 is

hydrogen. Preferably R_2 is hydrogen or alkyl of 1 to about 3 carbon atoms. Preferably T is $-C(=NR_7)NHR_8$. R_7 and R_8 are more preferably hydrogen.

According to an alternate embodiment of this aspect of the present invention, provided are compounds of formula I where E is phenyl. Preferably R_3 and R_{4b} are hydrogen and R_{4a} is hydrogen or methyl or q, R_3 and R_{4a} are selected together as set forth in paragraph (g) hereinabove.

According to a further embodiment of this aspect of this invention, provided are compounds of formula I selected from the compounds depicted in Figures 5A and 5B. Especially preferred compounds include Compounds A, C, D, F, L and M.

2. Preparation of the Preferred Compounds

Certain of the compounds of the present invention which have activity as inhibitors of matriptase or MTSP1 may be conveniently prepared by following the synthetic methods and techniques described in United States Patent Nos. 5,492,895; 5,534,498, 5,955,976; 5,646,195; 5,658,939; 5,696,231; 5,681,844; 5,703,208; 5,714,499; 5,731,413; 5,739,112; 5,770,600; 5,775,927; 5,883,077; 5,886,146; 6,034,215; 6,025,472; WO 00/05245; and Tamura, *et al.*, *Bioorganic & Medicinal Chemistry Letters* 9:2573-2578 (1999), the disclosures of which are incorporated by reference herein.

Figures 2A and 2B depict a synthetic scheme for the preparation of Compound No. 6 of Figure 1A.

Figures 4A and 4B depict a reaction scheme for the synthesis of and Examples 3 to 13 describe the preparation of certain compounds of present invention.

By substituting the appropriate reagents at various points in the reaction scheme depicted in Figures 4A to 4C and described in Examples 3 to 13, the compounds of formula I may be conveniently synthesized.

3. Selection of Preferred Compounds

According to one aspect of the present invention, compounds of the present invention are selected for their potency and selectivity in inhibiting serine protease activity of matriptase or MTSP1. As described in Examples A and B, and as is generally known, a target serine protease and its substrate are combined under assay conditions permitting reaction of the protease with its substrate. The assay is performed in the absence of a test compound, and in the presence of increasing concentrations of the test compound. The concentration of test compound at which 50% of the serine protease activity is inhibited by the test compound is the IC_{50} value (Inhibitory Concentration) or EC_{50} (Effective Concentration) value for that compound. Within a series or group of test compounds, those having lower IC_{50} or EC_{50} values are considered more potent inhibitors of the serine protease than those compounds having higher IC_{50} or EC_{50} values. The IC_{50} measurement is often used for more simplistic assays, whereas the EC_{50} is often used for more complicated assays, such as those employing cells.

Preferred compounds according to this aspect of the present invention have an IC_{50} value of 100nM or less as measured in an *in vitro* assay for inhibition of serine protease activity of matriptase or MTSP1 activity. Especially preferred compounds have an IC_{50} value of less than 30nM.

The test compounds also are evaluated for selectivity toward inhibiting serine protease activity of matriptase or MTSP1 in relation to other serine proteases (see Examples A and B). As described in the Examples, and as generally known, a test compound is assayed for its potency toward a panel of serine proteases and other enzymes and an IC_{50} value or EC_{50} value is determined for each test compound in each

assay system. A compound that demonstrates a low IC_{50} value or EC_{50} value for the target enzyme, e.g., the serine protease domain of matriptase or MTSP1, and a higher IC_{50} value or EC_{50} value for other enzymes within the test panel (e.g., tissue plasminogen activator, thrombin, Factor Xa), is considered to be selective toward the target enzyme. Generally, a compound is deemed selective if its IC_{50} value or EC_{50} value in the target enzyme assay is less than one half, preferably one-fifth and more preferably one order of magnitude less than the next smallest IC_{50} value or EC_{50} value measured in the selectivity panel of enzymes.

4. Pharmaceutical Compositions

In another aspect, the present invention encompasses pharmaceutical compositions prepared for storage or administration which comprise a therapeutically effective amount of a compound of the present invention in a pharmaceutically acceptable carrier.

The therapeutically effective amount of a compound of the present invention will depend on the route of administration, the type of mammal being treated, and the physical characteristics of the specific mammal under consideration. These factors and their relationship to determining this amount are well known to skilled practitioners in the medical arts. This amount and the method of administration can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize.

The therapeutically effective amount of the compound of the present invention can range broadly depending upon the desired effects and the therapeutic indication. Typically, dosages will be between about 0.01 mg/kg and 100 mg/kg body

weight, preferably between about 0.01 and 10 mg/kg body weight.

Pharmaceutically acceptable carriers for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). For example, sterile saline and phosphate-buffered saline at physiological pH may be used. Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition, antioxidants and suspending agents may be used. Id.

The pharmaceutical compositions of the present invention may be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions and suspensions for injectable administration; and the like. The dose and method of administration can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize.

When administration is to be parenteral, such as intravenous on a daily basis, injectable pharmaceutical compositions can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, or the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired,

absorption enhancing preparations (e.g., liposomes) may be utilized.

5. Utility

The compounds of the present invention have matriptase inhibitory activity.

The compounds of the present invention are active as inhibitors of matriptase and specifically bind matriptase. More particularly, preferred compounds bind to the serine protease domain of matriptase and inhibit its activity.

It is believed that these compounds will be useful in the prevention or treatment of cancerous conditions where that cancerous condition is exacerbated by the activity of matriptase.

Another use for the compounds of the present invention is to decrease progression of cancerous conditions and the concomitant degradation of the cellular matrix.

The compounds of the present invention are active as inhibitors of serine protease activity of matriptase or MTSP1 and specifically bind to the serine protease domain of matriptase or MTSP1. Accordingly, those compounds that contain sites suitable for linking to a solid/gel support may be used *in vitro* for affinity chromatography to purify matriptase from a sample or to remove matriptase from a sample using conventional affinity chromatography procedures. These compounds are attached or coupled to an affinity chromatography either directly or through a suitable linker support using conventional methods. See, *e.g.* Current Protocols in Protein Science, John Wiley & Sons (J.E. Coligan et al., eds, 1997) and Protein Purification Protocols, Humana Press (S. Doonan, ed., 1966) and references therein.

The compounds of the present invention having matriptase or MTSP1 serine protease inhibitory activity are

useful in *in vitro* assays to measure matriptase or MTSP1 activity and the ratio of complexed to uncomplexed matriptase or MTSP1 in a sample. These assays could also be used to monitor matriptase or MTSP1 activity levels in tissue samples, such as from biopsy or to monitor matriptase activities and the ratio of complexed to uncomplexed matriptase for any clinical situation where measurement of matriptase or MTSP1 activity is of assistance. An assay which determines serine protease activity in a sample could be used in combination with an ELISA which determines total amount of matriptase or MTSP1 (whether complexed or uncomplexed) in order to determine the ratio of complexed to uncomplexed matriptase.

Various animal models can be used to evaluate the ability of a compound of the present invention to reduce primary tumor growth or to reduce the occurrence of metastasis. These models can include genetically altered rodents (transgenic animals), transplantable tumor cells originally derived from rodents or humans and transplanted onto syngenic or immuno-compromised hosts, or they can include specialized models, such as the CAM model described below, designed to evaluate the ability of a compound or compounds to inhibit the growth of blood vessels (angiogensis) which is believed to be essential for tumor growth. Other models can also be utilized.

Appropriate animal models are chosen to evaluate the *in vivo* anti-tumor activity of the compounds described in this invention based on a set of relevant criteria. For example, one criterion might be expression of matriptase or MTSP1 and/or matriptase or MTSP1 mRNA by the particular tumor being examined. Two human prostate derived tumors that meet this criterion are the LnCap and PC-3 cell lines. Another criterion might be that the tumor is derived from a tissue

that normally expresses high levels of matriptase or MTSP1. Human colon cancers meet this criterion. A third criterion might be that growth and/or progression of the tumor is dependent upon processing of a matriptase or MTSP1 substrate (e.g., sc-u-PA). The human epidermoid cancer Hep-3 fits this criterion. Another criterion might be that growth and/or progression of the tumor is dependent on a biological or pathological process that requires matriptase or MTSP1 activity. Another criterion might be that the particular tumor induces expression of matriptase or MTSP1 by surrounding tissue. Other criteria may also be used to select specific animal models.

Once appropriate tumor cells are selected, compounds to be tested are administered to the animals bearing the selected tumor cells, and subsequent measurements of tumor size and/or metastatic spread are made after a defined period of growth specific to the chosen model.

The CAM model (chick embryo chorioallantoic membrane model), first described by L. Ossowski (J. Cell Biol. 107:2437-2445, 1988), provides another method for evaluating the anti-tumor and anti-angiogenesis activity of a compound.

Tumor cells of various origins can be placed on 10 day old CAM and allowed to settle overnight. Compounds to be tested can then be injected intravenously as described by Brooks, et al. (Methods in Molecular Biology 129:257-269, 1999). The ability of the compound to inhibit tumor growth or invasion into the CAM is measured 7 days after compound administration.

When used as a model for measuring the ability of a compound to inhibit angiogensis, a filter disc containing angiogenic factors, such as basic fibroblast growth factor (bFGF) or vascular ediothelial cell growth factor (VEGF), is placed on a 10 day old CAM as described by Brooks, et al.

(Methods in Molecular Biology 129:257-269, 1999). After overnight incubation, compounds to be tested are then administered intravenously. The amount of angiogenesis is measured by counting the amount of branching of blood vessels 48 hours after the administration of compound (Methods in Molecular Biology 129:257-269, 1999).

The compounds of the present invention are useful *in vivo* for treatment of pathologic conditions which would be ameliorated by decreased serine protease activity of matriptase or MTSP1.

It is believed these compounds will be useful in decreasing or inhibiting metastasis, and degradation of the extracellular matrix in tumors and other neoplasms. These compounds will be useful as therapeutic agents in treating conditions characterized by pathological degradation of the extracellular matrix, including those described hereinabove in the Background and Introduction to the Invention.

The present invention includes methods for preventing or treating a condition in a mammal suspected of having a condition which will be attenuated by inhibition of serine protease activity of matriptase or MTSP1 comprising administering to said mammal a therapeutically effective amount of a compound which selectively inhibits serine protease activity of matriptase or MTSP1 or a pharmaceutical composition of the present invention.

The compounds of the present invention are administered *in vivo*, ordinarily in a mammal, preferably in a human. In employing them *in vivo*, the compounds can be administered to a mammal in a variety of ways, including orally, parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms.

In practicing the methods of the present invention, the compounds of the present invention are administered alone or in combination with one another, or in combination with other therapeutic or *in vivo* diagnostic agents.

As is apparent to one skilled in the medical art, a "therapeutically effective amount" of the compounds of the present invention will vary depending upon the age, weight and mammalian species treated, the stage of the disease or pathologic condition being treated, the particular compounds employed, the particular mode of administration and the desired effects and the therapeutic indication. Because these factors and their relationship to determining this amount are well known in the medical arts, the determination of therapeutically effective dosage levels, the amount necessary to achieve the desired result of inhibiting matriptase or MTSP1 serine protease activity, will be within the ambit of one skilled in these arts. Typically, administration of the compounds of the present invention is commenced at lower dosage levels, with dosage levels being increased until the desired effect of inhibiting matriptase or MTSP1 activity to the desired extent is achieved, which would define a therapeutically effective amount. For the compounds of the present invention such doses are between about 0.01 mg/kg and about 100 mg/kg body weight, preferably between about 0.01 and about 10 mg/kg body weight.

6. Use of rMAP to Determine Matriptase or MTSP1 Inhibitor Activity

Examples 1 and 2 describe cloning and expression of a recombinant serine protease domain derived from matriptase or MTSP1 ("rMAP") using recombinant methods. Example A describes the use of rMAP in an amidolytic assay to test compounds for activity in inhibiting serine protease activity of matriptase or MTSP1. Suitable recombinant

serine protease domains according to the present invention include peptides which comprise SEQ. ID. NO. 2 or an amino acid sequence having serine protease activity and at least about 80% sequence identity to SEQ. ID. NO. 2. Such sequences include allelic variants and sequences having single nucleotide polymorphisms, but which result in peptides having serine protease activity.

Sequence identity of an amino acid sequence to a reference amino acid sequence (such as SEQ. ID. NO. 2) may be determined by techniques known to those skilled in the art. In making a determination regarding sequence identity, an alignment of the peptides to be compared is made. Such an alignment may be made manually or using certain algorithms developed for such use which are conventionally used. A comparison of residues occupying analogous positions is then made and the number of identical residues is scored.

Peptides which comprise a recombinant serine protease domain derived from matriptase or MTSP1 are useful in screening compounds for activity in inhibiting serine protease activity of matriptase or MTSP1. Use of one such peptide (rMAP) in an amidolytic assay is described in Example A.

To assist in understanding, the present invention will now be further illustrated by the following examples. These examples as they relate to this invention should not, of course, be construed as specifically limiting the invention and such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art and which are considered to fall within the scope of the invention as described herein and hereinafter claimed.

Examples

Example 1

Isolation and Cloning of Matriptase

A. Cell type and growth of cells

Human prostate adenocarcinoma cell line, PC-3, was purchased from ATCC (catalog number CRL-1435; Manassas, VA). The cells were cultured at 37°C, 5% CO_2 in Ham's F-12K growth medium (catalog number 9077; Irvine) supplemented with 2mM. L-glutamine and 10% fetal bovine serum. All subsequent cell manipulations were carried out according to the manufacturer's instructions. PC-3 cells were allowed to grow to about 90% confluence, and were then washed briefly with 1X phosphate buffered saline.

B. Isolation of total RNA, and purification and enrichment of $polyA^+$ RNAs

PC-3 cells were lysed in Trizol reagent (catalog number 15596; Life Technologies, Rockville, MD) and total RNA was isolated according to the manufacturer's protocol. The concentration of total RNA was estimated from absorbance reading at 260 nm. $PolyA^+$ RNAs were purified and enriched using oligo-dT beads (catalog number 70061; Oligotex, Qiagen, Valencia, CA).

C. Reverse-transcription and polymerase chain reaction (PCR)

PC-3-derived $polyA^+$ RNAs were converted to single-stranded cDNA (sscDNA) by reverse transcription using ProSTAR first-strand RT-PCR kit (catalog number 200420; Stratagene, La Jolla, CA) and SuperScript II RNase H^- reverse transcriptase (catalog number 18064-022; Life Technologies). After reverse transcription, an aliquot of PC-3 sscDNA (4 µL) was subjected to PCR using 2mM each of the sense and anti-sense degenerate oligonucleotide primers and *Taq* polymerase (catalog number 201203; Qiagen). Total reaction

volume was 100 μL. The sequence of the sense primer was 5'-TGGRT(I)VT(I)WS(I)GC(I)RC(I)CAYTG-3' (SEQ. ID. NO. 3), and that of the anti-sense was 5'-(I)GG(I)CC(I)CC(I)SWRTC(I)CCYT(I)RCA(I)GHRTC-3' (SEQ. ID. NO. 4), where R=A,G; V=G,A,C; W=A,T; S=G,C; Y=C,T; H=A,T,C. The primer sequences correspond to two highly conserved regions in all (chymo)trypsin-like serine proteases and amplify PCR products ranging from approximately 400 to 500 base pairs.

D. Clone screening and sequencing

The PCR products were separated on a 2% agarose gel and purified using a gel extraction kit (catalog number 28706; QIAquick gel extraction kit; Qiagen). The purified DNA fragments were ligated into pCR2.1-TOPO (catalog number K4500-01; Invitrogen, Carlsbad, CA). After transformation into *E. coli* cells, plasmid DNAs were isolated and analyzed by digestion with *Eco*RI restriction enzyme. Clones that had insert DNAs were further characterized by sequencing using a fluorescent dye-based DNA sequencing method (catalog number 4303149; BigDye terminator cycle sequencing kit with Ampli*Taq* DNA polymerase; Perkin Elmer, Lincoln, CA). A total of 31 clones were sequenced and analyzed. All sequences were analyzed by a multiple nucleotide sequence alignment algorithm (**blastn**) (www.ncbi.nlm.nih.gov/blast) to identify identical or closely related cDNAs deposited in GenBank (NCBI, Bethesda, MD). Those that did not show significant homology were further analyzed using **blastx**, which compares the six-frame conceptual translation products of a nucleotide sequence (both strands) against a protein sequence database (SwissProt). Eight clones yielded identical cDNA fragments that encode a novel serine protease, whose sequence did not match 100% to any deposited sequence at that time. This novel serine protease was named

MTSP1. The closest match found was with the mouse epithin mRNA (85% identity; GenBank accession number AF042822) which encodes a membrane-type serine protease, and the human SNC19 mRNA (98% identity; GenBank accession number HSU204,28) which contains a partial sequence of MTSP1. MTSP1 was subsequently found to be identical to matriptase (GenBank accession number AF118224) whose sequence appeared in GenBank after the cDNA encoding MTSP1 had been cloned and sequenced at Corvas.

E. Rapid amplification of cDNA ends (RACE) and gene-specific amplification of MTSP1

To obtain a cDNA encoding the complete protease domain of MTSP1, both RACE and gene-specific amplification reactions were performed. Since the presence of the transcript was detected in prostate, a human prostate Marathon-Ready cDNA (catalog # 7418-1; Clontech) was used to isolate part of the cDNA encoding MTSP1. Marathon-Ready cDNAs are prepared to contain a known hybridization sequence at both the 5′ and 3′ ends of the sscDNA and are therefore specifically suited for the RACE reactions. The 3′ region of MTSP1 cDNA was successfully obtained by a 3′-RACE reaction using a gene specific primer, 5′-CACCCCTTCTTCAATGACTTCACCTTCG-3′ (SEQ. ID. NO. 5). The 5′ end of the MTSP1 protease domain was obtained by gene-specific amplification reaction using two MTSP1-specific primers, 5′-TACCTCTCCTACGACTCC-3′ (SEQ. ID. NO. 6) for the sense primer and 5′-GAGGTTCTCGCAGGTGGTCTGGTTG-3′ (SEQ. ID. NO. 7) for the antisense primer. The sequences for these two primers were obtained from the human SNC19 mRNA sequence. Both 3′-RACE reaction and gene-specific PCR produced DNA fragments that were >1 kbp in size. These fragments were subcloned into pCR2.1-TOPO. After transformation into *E. coli* cells, plasmid DNAs were

isolated and analyzed by digestion with *Eco*RI restriction enzyme. Clones that had insert DNAs were characterized by Southern blot analysis (using the internal cDNA fragment as probe) and by DNA sequence analysis.

F. PCR amplification of cDNA encoding full-length protease domain of MTSP1

To obtain a cDNA fragment encoding the entire protease domain of MTSP1, an end-to-end PCR amplification using gene-specific primers was used. The two primers used were: 5′-CTCGAGAAAAGAGTTGTTGGGGGCACGGATGCGGATGAG-3′ (SEQ. ID. NO. 8) for the 5′ end and 5′-GCGGCCGCACTATACCCCAGTGTTCTCTTTGATCCA-3′ (SEQ. ID. NO. 9) for the 3′ end. The 5′ primer contained the sequence (underlined) that encodes the start of the MTSP1 protease domain (VVGGTDADE) (SEQ. ID. NO. 10). The 3′ primer contained the stop codon (underlined) of MTSP1. A ~800-bp fragment was amplified, purified and subcloned into the *Pichia pastoris* expression vector, pPIC9K, resulting in pPIC9K-MTSP1.

G. Gene expression profile of MTSP1 in normal tissues, cancer cells and cancer tissues

To obtain information regarding the tissue distribution and gene expression level of MTSP1, the DNA insert from pPIC9K-MTSP1 was used to probe a blot containing RNA from 76 different human tissues (catalog number 7775-1; human multiple tissue expression (MTE) array; CLONTECH, Palo Alto, CA). Significant expression was observed in the colon (ascending, transverse and descending), rectum, trachea, esophagus and duodenum. Moderate expression levels were observed in the jejunum, ileum, ilocecum, stomach, prostate, pituitary gland, appendix, kidney, lung, placenta, pancreas, thyroid gland, salivary gland, mammary gland, fetal kidney, and fetal lung. Lower expression levels were seen in the spleen, thymus, peripheral blood leukocyte, lymph node, bone

marrow, bladder, uterus, liver, adrenal gland, fetal heart, fetal liver, fetal spleen, and fetal thymus. A significant amount of the MTSP1 transcript was also detected in colorectal adenocarcinoma cell line (SW480), Burkitt's lymphoma cell line (Daudi), and leukemia cell line (HL-60). RT-PCR of the MTSP1 transcript in several human primary tumors xenografted in athymic nude mice was performed using gene-specific primers. A high level of MTSP1 transcript was detected in colon adenocarcinoma (CX-1) and pancreatic adenocarcinoma (GI-103). Moderate levels were observed in another colon adenocarcinoma (GI-112), ovarian carcinoma (GI-102), lung carcinoma (LX-1), and breast carcinoma (GI-101). Another lung carcinoma (GI-117) expressed a low level of the MTSP1 transcript. A similar RT-PCR was performed to detect the presence of the MTSP1 transcript in PC-3 and LNCaP cell lines. Both cell lines expressed significant amounts of MTSP1 transcript.

H. Sequence analysis

All derived DNA and protein sequences were analyzed using MacVector (version 6.5; Oxford Molecular Ltd., Madison, WI). The cDNA encoding the protease domain of MTSP1 is composed of 726 base pairs which translate into a 241-amino acid protein sequence (rMAP). See Figures 3A to 3C.

Example 2

Production of Recombinant Serine Protease Domain of Matriptase or MTSP1 (rMAP)

A. Fermentation

The production of multi-milligram amounts of rMAP was carried out by fermentation in a BioFlo 3000 fermenter (New Brunswick Scientific, NJ) equipped with a 3.3 L capacity bioreactor using a SMD1168/pPIC9K:MTSP1 Sac SC1 clone. ZA001 complex media (10 g/L yeast extract, 20 g/L peptone,

40 g/L glycerol, 5 g/L ammonium sulfate, 0.2 g/L calcium sulfate dihydrate, 2 g/L magnesium sulfate heptahydrate, 2 g/L potassium sulfate, 25 g/L sodium hexametaposphate, 4.35 mL/L PTM1) was inoculated with 100 mL of an overnight culture of the *P. pastoris* transformant. The culture was supplemented with 50% glycerol by fed-batch phase and induced for 18-24 hours with methanol controlled at 0.025%.

B. Purification of Recombinant Serine Protease Domain of Matriptase or MTSP1 (rMAP)

The rMAP was secreted into the culture media, so the first step of the purification involved the removal of cells and cell debris by centrifugation at 5000 g for 30 minutes. The resulting supernatant was decanted, adjusted to pH 8.0 with 10N NaOH, and filtered through a SartoBran 300 0.45 + 0.2 μM capsule. This supernatant was concentrated to 1 L by ultrafiltration using a 10kDa ultrafiltration cartridge (NC SRT UF system with AG/Technologies UFP-10-C-5A filter), and the buffer was exchanged by crossflow filtration into 50mM tris-HCl, 50mM NaCl, 0.05% tween-80, pH 8.0 (buffer A). The filtration unit was rinsed once with 1 L buffer A which was combined with the concentrate.

The concentrated rMAP-containing solution was passed over a 150 mL benzamidine column that had been equilibrated with buffer A, at a flow rate of 8 mL/min. The column was washed with 3 column volumes of 50mM tris-HCl, 1.0M NaCl, 0.05% tween-80, pH 8.0 (buffer B) and eluted with 3 column volumes of 50mM tris-HCl, 1.0M L-arginine, 0.05% tween-80, pH 8.0 (buffer C). Fractions containing rMAP were identified by activity assay and pooled. This pooled material was concentrated to 10 mL using a JumboSep concentrator (Pall Gelman) and a 10kDa cutoff membrane. Once concentrated to 10 mL, the buffer was exchanged into 50mM Na_2HPO_4, 125mM NaCl, pH 5.5 (buffer D) and the volume

adjusted to 5-10 mL. The retentate was removed and the concentrator washed with buffer D which was added to the concentrate. The total sample volume was adjusted 15 mL.

The partially purified rMAP was passed through a 5 mL Q-Sepharose Fast Flow HiTrap column (Amersham-Pharmacia Biotech) pre-equilibrated with 15 mL of buffer D. The flow through was collected. The HiTrap column was washed with an additional 10 mL of buffer D. Both flow throughs were pooled, and the protein concentration was determined by measurement of OD_{280} (using an extinction coefficient of 2.012 mg/OD_{280}). Purified rMAP was then deglycosylated by the addition 0.1 µL of Endoglycosidase H (ProZyme, 5 U/mL) per mg of protein and incubating overnight at 4°C with gentle swirling.

The conductivity of the deglycosylated pool was adjusted to 2.0-3.0 mS/cm with Nanopure H_2O and the pH adjusted to 6.5 (200-300mL final volume). The rMAP was then further purified by anion exchange chromatography by loading directly onto a Pharmacia Akta Explorer system using a 7 mL Source 15Q anion exchange column (Amersham-Pharmacia Biotech). The protein was eluted in a buffer containing 50mM HEPES, pH 6.5 with a 0-0.33M NaCl gradient over 10 column volumes at a flow rate of 6 mL/min. Fractions containing protein were pooled, and benzamidine was added to a final concentration of 10mM. Protein purity was examined by SDS-PAGE and protein concentration determined by measurement of OD_{280} and use of a theoretical extinction coefficient of 2.012 mg/OD_{280}.

Example 3

Preparation of (R)-2-tert-Butyl-(S)-4-(3-cyano-benzyl)-3-formyl-oxazolidine-4-carboxylic acid benzyl ester (**2**)

A oven dried 1L round bottom flask containing a Teflon-coated magnetic stirring bar and a rubber septum was charged

with THF (225 mL) and cooled to −78°C. The reaction mixture was treated with KHMDS (75.5 mL of 0.5M in toluene, 37.8 mmol, 1.1 eq) and stirred for 20 minutes; then (*R*)-2-tert-butyl-3-formyl-oxazolidine-(*S*)-4-carboxylic acid benzyl ester (**1**) (Seebach et. al. Helv. Chim. Acta v70,(1987), pg. 1194-1216) (10 g, 34.3 mmol) dissolved in THF (50 mL) was added over a 30 minute period via syringe and syringe pump. Once the addition was complete, the reaction mixture was stirred for 2 hours at −78°C and treated with α-bromo-*m*-tolunitrile (8.75 g, 44.6 mmol, 1.3 eq) dissolved in THF (50 mL) via cannula over a 5 minute period. The reaction mixture was stirred for 2 hours at -78°C and then allowed to warm to ambient temperature over a 10 hour period (dry ice/isopropanol bath was allowed to melt). The reaction mixture was poured into a vigorously stirred mixture of ice/$NH_4Cl_{(sat)}$ (100 mL) and stirred for 20 minutes. The biphasic mixture was transferred to a separatory funnel and the phases were separated. The aqueous phase was re-extracted with ethyl acetate (3 x 75 mL) and the combined organic phases were washed with 0.5N HCl (100 mL), H_2O (50 mL), $NaHCO_{3(sat)}$ (50 mL), H_2O (50 mL), brine (100 mL), dried ($MgSO_4$), filtered and concentrated to give a thick yellow oil. The crude material was triturated with 30% ethyl acetate/hexane, filter and concentrated to give a yellow oil which was purified by flash chromatography (SiO_2, 40% ethyl acetate/hexane for elution) to give the title compound as a white crystalline solid (10.3 g, 74%).

(*Other appropriate electrophiles (i.e.* α-bromo-*p*-tolunitrile*) can be substituted in this step to obtain other compounds of formula I.)*

Example 4

Preparation of (*S*)-Amino-2-(3-cyano-benzyl)-3-hydroxy-propionic acid benzyl ester (**3**):

A 1L round bottom flask equipped with a reflux condenser and a Teflon-coated magnetic stirring bar was charged with compound **2** (30 g, 73.8 mmol) and MeOH (1 L). 6N HCl (45 mL) was added and the slightly cloudy reaction mixture was heated to a gentle reflux with stirring for 48 hours. The reaction mixture was cooled to ambient temperature and concentrated to dryness to give a white colorless solid, which was dissolved in H_2O (300 mL) and extracted with CH_2Cl_2 (50 mL). The aqueous phase was concentrated to give the title compound as a white colorless solid (22.7 g, 89%).

Example 5

Preparation of (*S*)-Cyano-benzyl)-3-hydroxy-2-methoxycarbonylamino-propionic acid (**4**)

A 250 mL round bottom flask containing a Teflon-coated magnetic stirring bar and a rubber septum was charged with compound (**3**) (10 g, 28.8 mmol) and CH_2Cl_2 (250 mL). Methyl chloroformate (3.54 g, 37.5 mmol, 1.3 eq) was added to the reaction mixture and the mixture was stirred for 48 hours. H_2O (100 mL) was added and the mixture was stirred for 20 minutes. The biphasic mixture was transferred to a separatory funnel and the phases were separated. The aqueous phase was re-extracted with CH_2Cl_2 (3 x 50 mL) and the combined organic phases were washed with H_2O (50 mL), brine (50 mL), dried ($MgSO_4$), filtered and concentrated to give a slightly yellow oil. The crude material was purified by flash chromatography (SiO_2, 40% ethyl acetate/hexane for elution) to give the title compound as colorless oil (9.17g, 86%).

(Other suitable capping groups (acyl chlorides, isocyanates, sulfonyl chlorides) can be substituted for the chloroformate in this step to obtain other compounds of formula I.)

Example 6

Preparation of (*R*)-4-(3-Cyano-benzyl)-4-methoxycarbonylamino-pent-(*E*)-2-enedioic acid-1-tert-butyl ester (**5**)

An oven dried 500 mL round bottom flask containing a Teflon-coated magnetic stirring bar and a rubber septum was charged with CH_2Cl_2 (200 mL) and cooled to −78°C. Oxalyl chloride (4.1 g, 33 mmol, 1.5 eq) was added to the reaction mixture and was stirred for 10 minutes at −78°C. DMSO (3.4 g, 43 mmol, 2 eq) was added dropwise over a 15 minute period and stirred for an additional 15 minutes at −78°C. Compound **4** (8 g, 22 mmol) dissolved in CH_2Cl_2 (50 mL) was added dropwise via syringe and syringe pump over a 30 minute period. Once the addition was complete, the reaction mixture was stirred at −78°C for 30 minutes and then treated with triethylamine (10 g, 109 mmol, 5 eq). The reaction mixture was allowed to warm to ambient temperature and stirred for an additional 20 minutes. Water (50 mL) was added and stirred vigorously for 20 minutes. The biphasic mixture was transferred to a separatory funnel and the phases were separated. The aqueous phase was re-extracted with ethyl acetate (3 x 50 mL). The combined organic phases were washed with 0.5N HCl (100 mL), H_2O (100 mL), $NaHCO_{3(sat)}$ (100 mL), brine (100 mL), dried ($MgSO_4$), and filtered into a round bottom flask. The filtrate was treated with (*tert*-butoxycarbonyl-methylene)triphenylphosphorane (12.3 g, 33 mmol, 1.5 eq) and stirred at ambient temperature for 18 hours. The reaction mixture was concentrated and the crude material was

dissolved in a minimal amount of CH_2Cl_2 (~25 mL) and purified by flash chromatography (SiO_2, loaded with CH_2Cl_2, 40% ethyl acetate/hexane for elution) to give the title compound as a colorless solid (7.4 g, 73%).

Example 7

Preparation(*R*)-2-(3-Cyano-benzyl)-2-methoxycarbonylamino-pentanedioic acid-5-*tert*-butyl ester methoxycarbonylamino-pentanoic acid *tert*-butyl ester **(6)**

To a round bottom flask containing a suspension of Pd-$BaSO_4$ (231 mg, 51% by wt) and MeOH (25 mL) was added compound **5** (455 mg, 0.98 mmol). The heterogeneous mixture was evacuated and the flask was back filled with H_2 several times before being stirred for 72 hours (1 atm of H_2, balloon). The Pd catalyst was filtered off and rinsed several times with MeOH. The filtrate was concentrated to give the title compound as a colorless solid (405 mg, 89%).

Example 8

Preparation of 4-(Benzyloxycarbonylmethyl-carbamoyl)-(*R*)-5-(3-cyano-phenyl)-4-methoxycarbonylamino-pentanoic acid *tert*-butyl ester (**7**)

A round bottom flask containing a Teflon-coated magnetic stirring bar and a rubber septum was charged with compound **6** (387 mg, 1.03 mmol), EDC (296 mg, 1.54 mmol, 1.5 eq), HOBt (173 mg, 1.13 mmol, 1.1 eq), H-Gly(OBzl)•HCl (270 mg, 1.34 mmol, 1.3 eq), and acetonitrile (10 mL). The reaction mixture was treated with DIEA (0.477 mL, 2.67 mmol, 2.6 eq) and stirred for 18 hours. Acetonitrile was removed under vacuum and the crude material was treated with a mixture of ethyl acetate and H_2O and transferred to a separatory funnel. The phases were separated and the aqueous phase was re-extracted with ethyl acetate (3 x 10 mL). The combined organic phases were washed with 0.5N HCl

(10 mL), H_2O (10 mL), $NaHCO_{3(sat)}$ (10 mL), brine (10 mL), dried (MgSO4), filtered and concentrated to give that title product as a colorless, thick oil (527 mg, 98%). *(Other suitable amino acids (ie. β-alanine(OBzl), proline(OBzl)) can be substituted in this step to obtain other compounds of formula I.)*

Example 9

Preparation of 4-(Carboxymethyl-carbamoyl)-5-(3-cyano-phenyl)-(*R*)-4-methoxycarbonylamino-pentanoic acid *tert*-butyl ester (**8**)

To a round bottom flask containing a suspension of Pd-$BaSO_4$ (47 mg) and MeOH (10 mL) was added compound **7** (527 mg, 1.01 mmol). The reaction flask was evacuated and back filled with H_2 (1 atm, balloon) several times before being stirred for 30 minutes (the reaction was followed by TLC). The Pd catalyst was filtered off and rinsed several times with MeOH. The filtrate was concentrated to give the title compound a colorless solid (383 mg, 88%).

Example 10

Preparation of (*R*)-5-(3-Cyano-phenyl)-4-({[2-ethoxy-1-(N-nitrocarbamimidoyl)-piperidin-(*S*)-3-ylcarbamoyl]-methyl}carbamoyl-4-methoxycarbonlyamino-pentanoic acid *tert*-butyl ester (**9**)

A round bottom flask containing a Teflon-coated magnetic stirring bar and a rubber septum was charged with compound **8** (367 mg, 0.847 mmol), EDC (325 mg, 1.69 mmol, 2 eq), HOBt (194 mg, 1.27 mmol, 1.5 eq), HCl•H_2N-Arg(NO_2)-EtAminal (453 mg, 1.69 mmol, 2 eq), and acetonitrile (15 mL). The reaction mixture was treated with DIEA (0.767 mL, 4.40 mmol, 5.2 eq) and stirred for 18 hours and concentrated. Ethyl acetate (10 mL) and H_2O (10 mL) were added. The biphasic mixture was transferred to a separatory

funnel and the phases were separated. The aqueous phase was re-extracted with ethyl acetate (3 x 10 mL) and the combined organic phases were washed with 0.5N HCl (10 mL), H_2O (10 mL), $NaHCO_{3(sat)}$ (10 mL), brine (10 mL), and then dried ($MgSO_4$), filtered and concentrated to give that title product as a light yellow solid (502 mg, 92%).

Example 11

Preparation of (*R*)-4-({[2-Ethoxy-1-(N-nitrocarbamimidoyl)-piperidin-(*S*)-3-ylcarbamoyl]-methyl}-carbamoyl)-5-[3-(N-hydroxycarbamoyl)-phenyl]-4-carbonylamino-pentanoic acid tert-butyl ester (**10**)

A round bottom flask containing a Teflon-coated magnetic stirring bar and a rubber septum was charged with compound **9** (483 mg, 0.747 mmol), HCl•H_2NOH (519 mg, 7.47 mmol) and MeOH (20 mL). The homogenous reaction mixture was treated with *N*-methylmorpholine (1.65 mL, 14.9 mmol) and stirred for 48 hours. The reaction mixture was concentrated to give a yellow solid which was dissolved in H_2O/acetonitrile (10 mL/ 5 mL) and which was purified by preparatory HPLC (C_{18}, acetonitrile/H_2O (0.1%TFA gradient for elution)) to give the title compound as a white fluffy solid (363 mg, 53%) after lyophilization.

Example 12

(*R*)-4-{[1-Carbamimidoyl-2-ethoxy-piperidi-(*S*)-3-ylcarbamoyl)-methyl]-carbamoyl}-5-(3-diaminomethyl-phenyl-4-methoxycarbonylamino-pentanoic acid *tert*-butyl ester (**11**)

To a high pressure reaction vessel containing a suspension of 10% Pd-C (11 mg, 20% by wt) and a solution of EtOH/AcOH/H_2O (10:2:1) (10 mL) was added compound **10** (55 mg, 8.1×10^{-5} mol). The reaction vessel was evacuated and back filled with H_2 (50 psi) several times before being stirred for 48 hours (the reaction was followed by mass

spectroscopy). The Pd catalyst was filtered off and rinsed several times with MeOH. The filtrate was concentrated to give a colorless solid (45 mg, 89%), which was purified by preparatory HPLC (C_{18}, acetonitrile/H_2O (0.1%TFA gradient for elution)) to give the title compound as a white fluffy solid (21 mg) after lyophilization.

Example 13

Preparation of (*R*)-5-(3-diaminomethyl-phenyl)-4-{[1-formyl-(*S*)-4-guanidino-butylcarbamoyl-methyl]-carbamoyl}-4-methoxycarbonylamino-pentanoic acid *tert*-butyl ester (**12**)

A round bottom flask was charged with compound **11** (12.5 mg, 0.015 mmol) and 6N HCl (0.4 mL). The reaction mixture was stirred for 0.5 hours (monitored by HPLC), diluted with H_2O (5 mL) and purified by preparative chromatography (reverse phase C_{18}, acetonitrile/H_2O (.1%TFA)) to give the title compound as a white fluffy solid (8.4 mg) after lyophilization.

Example A

Amidolytic Assay for Determining Inhibition of Serine Protease Activity of Matriptase or MTSP1

The ability of the compounds of the present invention to act as inhibitors of rMAP catalytic activity was assessed by determining the inhibitor-induced inhibition of amidolytic activity by the MAP, as measured by IC_{50} values.

The assay buffer was HBSA (10mM Hepes, 150mM sodium chloride, pH 7.4, 0.1% bovine serum albumin). All reagents were from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Two IC_{50} assays (a) one at either 30-minutes or 60-minutes (a 30-minute or a 60-minute preincubation of test compound and enzyme) and (b) one at 0-minutes (no preincubation of test compound and enzyme) were conducted.

For the IC_{50} assay at either 30-minutes or 60-minutes, the following reagents were combined in appropriate wells of a Corning microtiter plate: 50 microliters of HBSA, 50 microliters of the test compound, diluted (covering a broad concentration range) in HBSA (or HBSA alone for uninhibited velocity measurement), and 50 microliters of the rMAP (Corvas International) diluted in buffer, yielding a final enzyme concentration of 250pM as determined by active site filtration. Following either a 30-minute or a 60-minute incubation at ambient temperature, the assay was initiated by the addition of 50 microliters of the substrate S-2765 (N-α-Benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-p-nitroaniline dihydrochloride; DiaPharma Group, Inc.; Franklin, OH) to each well, yielding a final assay volume of 200 microliters and a final substrate concentration of 100μM (about 4-times K_m). Before addition to the assay mixture, S-2765 was reconstituted in deionized water and diluted in HBSA. For the IC_{50} assay at 0 minutes; the same reagents were combined: 50 microliters of HBSA, 50 microliters of the test compound, diluted (covering the identical concentration range) in HBSA (or HBSA alone for uninhibited velocity measurement), and 50 microliters of the substrate S-2765. The assay was initiated by the addition of 50 microliters of rMAP. The final concentrations of all components were identical in both IC_{50} assays (at 30- or 60- and 0-minute).

The initial velocity of chromogenic substrate hydrolysis was measured in both assays by the change of absorbance at 405 nm using a Thermo Max® Kinetic Microplate Reader (Molecular Devices) over a 5 minute period, in which less than 5% of the added substrate was utilized. The concentration of added inhibitor, which caused a 50% decrease in the initial rate of hydrolysis was defined as

the respective IC_{50} value in each of the two assays (30- or 60-minutes and 0-minute).

Results for certain compounds of the present invention are reported in Table I.

Example B

In vitro enzyme assays for specificity determination

The ability of compounds to act as a selective inhibitor of matriptase activity was assessed by determining the concentration of test-compound which inhibited the activity of matriptase by 50%, (IC_{50}) as described in Example A, and comparing IC_{50} value for matriptase to that determined for all or some of the following serine proteases: thrombin, recombinant tissue plasminogen activator (rt-PA), plasmin, activated protein C, chymotrypsin, factor Xa and trypsin.

The buffer used for all assays was HBSA (10mM HEPES, pH 7.5, 150mM sodium chloride, 0.1% bovine serum albumin).

The assay for IC_{50} determinations was conducted by combining in appropriate wells of a Corning microtiter plate, 50 microliters of HBSA, 50 microliters of the test compound at a specified concentration (covering a broad concentration range) diluted in HBSA (or HBSA alone for V_0 (uninhibited velocity) measurement), and 50 microliters of the enzyme diluted in HBSA. Following a 30 minute incubation at ambient temperature, 50 microliters of the substrate at the concentrations specified below were added to the wells, yielding a final total volume of 200 microliters. The initial velocity of chromogenic substrate hydrolysis was measured by the change in absorbance at 405 nm using a Thermo Max® Kinetic Microplate Reader over a 5 minute period in which less than 5% of the added substrate was utilized. The concentration of added inhibitor which

caused a 50% decrease in the initial rate of hydrolysis was defined as the IC_{50} value.

Thrombin (fIIa) Assay

Enzyme activity was determined using the chromogenic substrate, Pefachrome t-PA (CH_3SO_2-D-hexahydrotyrosine-glycyl-L-Arginine-p-nitroaniline, obtained from Pentapharm Ltd.). The substrate was reconstituted in deionized water prior to use. Purified human α-thrombin was obtained from Enzyme Research Laboratories, Inc. The buffer used for all assays was HBSA (10mM HEPES, pH 7.5, 150mM sodium chloride, 0.1% bovine serum albumin).

IC_{50} determinations were conducted where HBSA (50 µL), α-thrombin (50 µL) (the final enzyme concentration is 0.5 nM) and inhibitor (50 µL) (covering a broad concentration range), were combined in appropriate wells and incubated for 30 minutes at room temperature prior to the addition of substrate Pefachrome-t-PA (50 µL) (the final substrate concentration is 250µM, about 5 times Km). The initial velocity of Pefachrome t-PA hydrolysis was measured by the change in absorbance at 405 nm using a Thermo Max® Kinetic Microplate Reader over a 5 minute period in which less than 5% of the added substrate was utilized. The concentration of added inhibitor which caused a 50% decrease in the initial rate of hydrolysis was defined as the IC_{50} value.

Factor Xa

Factor Xa catalytic activity was determined using the chromogenic substrate S-2765 (N-benzyloxycarbonyl-D-arginine-L-glycine-L-arginine-p-nitro aniline), obtained from DiaPharma Group (Franklin, OH). All substrates were reconstituted in deionized water prior

to use. The final concentration of S-2765 was 250µM (about 5-times Km). Purified human Factor X was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN) and Factor Xa (FXa) was activated and prepared from it as described [Bock, P.E., Craig, P.A., Olson, S.T., and Singh, P. *Arch. Biochem. Biophys.* 273:375-388 (1989)]. The enzyme was diluted into HBSA prior to assay in which the final concentration was 0.25nM.

<u>Recombinant tissue plasminogen activator (rt-PA) Assay</u>

rt-PA catalytic activity was determined using the substrate, Pefachrome t-PA (CH_3SO_2-D-hexahydrotyrosine-glycyl-L-arginine-p-nitroaniline, obtained from Pentapharm Ltd.). The substrate was made up in deionized water followed by dilution in HBSA prior to the assay in which the final concentration was 500 micromolar (about 3-times Km). Human rt-PA (Activase®) was obtained from Genentech Inc. The enzyme was reconstituted in deionized water and diluted into HBSA prior to the assay in which the final concentration was 1.0nM.

<u>Plasmin Assay</u>

Plasmin catalytic activity was determined using the chromogenic substrate, S-2366 [L-pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline hydrochloride], which was obtained from DiaPharma group. The substrate was made up in deionized water followed by dilution in HBSA prior to the assay in which the final concentration was 300 micromolar (about 2.5-times Km). Purified human plasmin was obtained from Enzyme Research Laboratories, Inc. The enzyme was diluted into HBSA prior to assay in which the final concentration was 1.0nM.

Activated Protein C (aPC) Assay

aPC catalytic activity was determined using the chromogenic substrate, Pefachrome PC (delta-carbobenzloxy-D-lysine-L-prolyl-L-arginine-p-nitroailine dihydrochloride), obtained from Pentapharm Ltd.). The substrate was made up in deionized water followed by dilution in HBSA prior to the assay in which the final concentration was 400 micromolar (about 3-times Km). Purified human aPC was obtained from Hematologic Technologies, Inc. The enzyme was diluted into HBSA prior to assay in which the final concentration was 1.0nM.

Chymotrypsin Assay

Chymotrypsin catalytic activity was determined using the chromogenic substrate, S-2586 (methoxy-succinyl-L-arginine-L-prolyl-L-tyrosyl-p-nitroanilide), which was obtained from DiaPharma Group. The substrate was made up in deionized water followed by dilution in HBSA prior to the assay in which the final concentration was 100 micromolar (about 9-times Km). Purified (3X-crystallized; CDI) bovine pancreatic alpha-chymotrypsin was obtained from Worthington Biochemical Corp. The enzyme was reconstituted in deionized water and diluted into HBSA prior to assay in which the final concentration was 0.5nM.

Trypsin Assay

Trypsin catalytic activity was determined using the chromogenic substrate, S-2222 (benzoyl-L-isoleucine-L-glutamic acid-[gamma-methyl ester]-L-arginine-p-nitroanilide), which was obtained from DiaPharma Group. The substrate was made up in deionized water followed by dilution in HBSA prior to the assay in which the final concentration was 250 micromolar (about 4-times Km). Purified (3X-crystallized; TRL3) bovine

pancreatic trypsin was obtained from Worthington Biochemical Corp. The enzyme was reconstituted in deionized water and diluted into HBSA prior to assay in which the final concentration was 0.5nM.

Table I lists the determined IC_{50} values for certain of the enzymes listed above for certain compounds depicted in Figure 5A and 5B and demonstrate the high degree of specificity for the inhibition of matriptase compared to the other serine proteases.

TABLE I

Compound	Matriptase IC_{50} 30 Min	FXa IC_{50} 30 Min	Thrombin IC_{50} 30 Min	Plasmin IC_{50} 30 Min	Thypsin IC_{50} 30 Min
A	A	E	E	D	C
B	A	E	D	E	E
C	A	E	E	D	D
D	A	E	E	E	E
E	A	E	F	F	E
F	A	E	E	D	D
G	A	E	E	D	A
H	A	F	E	F	E
I	A	D	E	E	D
J	A	E	E	E	D
K	A	E	F	F	F
L	A	E	F	F	E
M	A	D	F	E	C

A = less than 100 nM

B = 100 to 250 nM

C = 250 to 500 nM

D = 500 to 2500 nM

E = Greater than 2500 nM

F = Inactive

NT = Not Tested